Synergistic Function of E2F7 and E2F8 is Essential for Embryo Development

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Introduction

The E2F transcription factor family regulates fundamental cellular processes by controlling the transcription of a plethora of genes involved in proliferation, differentiation and apoptosis. E2F activity is complex. Based on function studies, the originally identified E2F family members (E2F1-6) can be divided into two subclasses, transcription activators and repressors. Members of the activator subclass, consisting of E2F1, E2F2 and E2F3, bind E2F targets and induce gene expression. The repressor subclass composed of E2F4, E2F5 and E2F6, suppresses expression of their targets (Cam and Dynlacht, 2003). Hetero-dimerization with DP proteins (dimerization partners) is required for E2F function (Trimarchi and Lees, 2002). Recently, the complexity of this family was further extended by the identification of two novel family members, named as E2F7 and E2F8 (de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004; Maiti et al., 2005; Christensen et al., 2005; Logan et al., 2005).

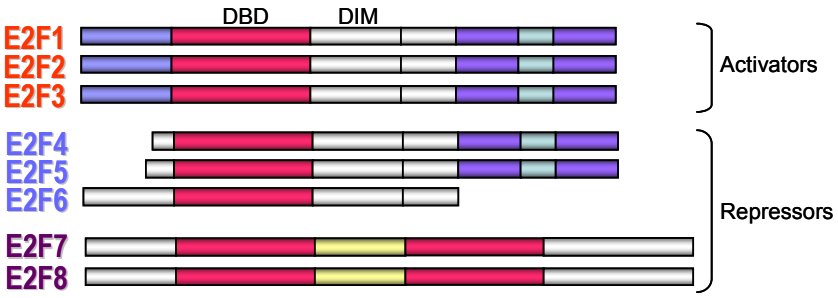


Figure 1. Schematic representation of the E2F transcription factors. A highly conserved DNA binding domain (DBD) is the hallmark of the E2F family of transcription factors. Different from other E2Fs, E2F7 and E2F8 have two distinct DBDs. DP dimerization domain (DIM) common to E2F1-6 is involved in dimerizing with DP, but is absent in E2F7 and E2F8.

E2F7 and E2F8 are conserved in mice and humans (Li et al., 2008). These two novel E2F family members share unique characteristics, and have several salient features that distinguish them from other family members. The most notable featureof E2F7 and E2F8 is that they each possess two DNA binding domains (E2F1-6 only have one DBD) and lack of DP dimerization domain (DIM), which allows them to function in a DP-independent manner

(Figure 1). Other features include their abilities to homo-dimerize with themselves and hetero-dimerize with each other, to silence target gene expression, and to block cellular proliferation when overexpressed *in vitro* (de Bruin et al., 2003; Di Stefano et al., 2003; Maiti et al., 2005). However, as the most recently identified and the least studied E2Fs, *in vivo* functions of E2F7 and E2F8 remain unknown.

The molecular understanding of how proliferation, differentiation and apoptosis is regulated in normal and cancer cells remains as a cornerstone to the development of any future treatment of cancer patients in humans. While E2Fs have been shown to play a key role in controlling these events, our understanding has been hampered by the fact that it was only very recently that E2F7 and E2F8, the last two family members contributing to "E2F" activity, were identified. In this regard, the complete understanding of the E2F activity will only be possible until physiological functions of E2F7 and E2F8 are fully revealed.

Results

Genetic Ablation of E2f7 and E2f8 in vivo Leads to Embryonic Lethality and Massive Apoptosis in the Fetus.

To investigate E2F7 and E2F8 function *in vivo,* we utilized knockout strategies to disrupt *E2f7* and *E2f8* genes in mice. Intercrosses between heterozygous *E2f7* or *E2f8* animals (*E2f7*$^{+/-}$ and *E2f8*$^{+/-}$, respectively) resulted

Table 1: Genotypic analysis of embryos during development

	E2f7$^{+/+}$			*E2f7*$^{+/-}$			*E2f7*$^{-/-}$			total
	E2f8$^{+/+}$	*E2f8*$^{+/-}$	*E2f8*$^{-/-}$	*E2f8*$^{+/+}$	*E2f8*$^{+/-}$	*E2f8*$^{-/-}$	*E2f8*$^{+/+}$	*E2f8*$^{+/-}$	*E2f8*$^{-/-}$	
E9.5	6	16	29	16	72(2)	53(1)	12	58(1)	33	299
expected	*5*	*28*	*22*	*19*	*75*	*56*	*13*	*47*	*34*	
E10.5	9	21	13	7(1)	45	28(1)	4(1)	28(1)	6(7)[a]	172
expected	*7*	*20*	*13*	*16*	*43*	*28*	*8*	*23*	*14*	
E11.5	-	6	4	2	15(2)	14(2)	2(1)	8(1)	0(5)[b]	62
expected	*-*	*5*	*5*	*3*	*16*	*13*	*3*	*10*	*8*	
E12.5	-	-	3	-	4	17(2)	-	3	0(9)[b]	38
expected	*-*	*-*	*5*	*-*	*4*	*15*	*-*	*4*	*10*	
P0	7	22	16	24	45	17	5	18	0[b]	154
expected	*8*	*18*	*10*	*18*	*39*	*21*	*10*	*21*	*11*	

() number of dead embryos recovered; Exact binomial test: [a] significant (p=0.0015), [b] highly significant (p<0.0007).

in viable single knockout offspring (SKO, *E2f7*$^{-/-}$ or *E2f8*$^{-/-}$) that appeared normal, were fertile and lived to old age (data not shown). We then explored functional redundancy between E2F7 and E2F8 by examining the biological consequence of ablating both simultaneously. To this end, we intercrossed *E2f7*$^{+/-}$*E2f8*$^{+/-}$ and *E2f7*$^{+/-}$*E2f8*$^{-/-}$ animals and analyzed the resulting offspring. Whereas *E2f7*$^{-/-}$*E2f8*$^{+/-}$ and *E2f7*$^{+/-}$*E2f8*$^{-/-}$ pups were born at the expected Mendelian ratios, no double knockout pups (DKO, *E2f7*$^{-/-}$*E2f8*$^{-/-}$) were found in newborn litters (Table 1, P0), demonstrating that at least one allele of *E2f7* or *E2f8* is required for embryo development and viability.

Analysis at earlier stages of embryonic development revealed that 6 out of 13 DKO embryos were alive at embryonic day 10.5 (E10.5) and none were alive at E11.5 (Table 1). All DKO embryos identified at E9.5 had a beating heart, but these embryos were noticeably smaller than wild type littermates (Figure 2a, top panels). Macroscopic inspection of embryos did not reveal other obvious defects at this stage of development. By E10.5, however, vascular defects were observed in the embryo proper, which were characterized by large dilated blood vessels associated with multifocal hemorrhages (Figure 2a, bottom panels).

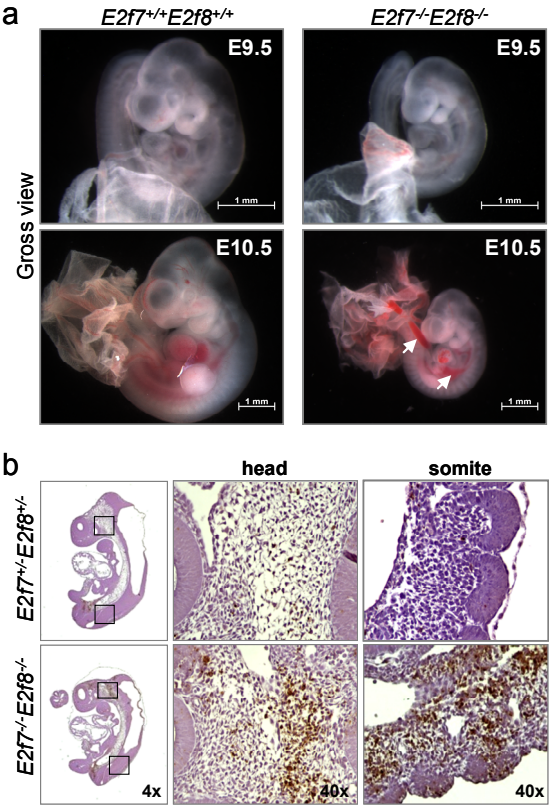


Figure 2. Global deletion of *E2f7* and *E2f8* results in developmental delay, vascular defects and widespread apoptosis *in vivo*. (a) Gross pictures of DKO embryos at E9.5 (top panels) and E10.5 (bottom panels). The vascular defects in E10.5 *E2f7*$^{-/-}$*E2f8*$^{-/-}$ embryo are indicated by arrows. (b) Embryo sections with the indicated genotypes were analyzed by TUNEL assays. Far left panels: low magnification pictures of whole embryos. Right panels: high magnification pictures of the boxed areas from the low magnification pictures. Apoptotic cells were labeled in brown color.

We then examined proliferation and apoptosis in DKO embryos more closely. When assessed by immunohistochemistry (IHC) using antibodies specific for the S-phase marker BrdU, we observed no detectable difference in proliferation between DKO and wild type embryos (data not shown). We did find, however, a massive number of cells labelled by TdT-mediated dUTP nick end-labelling (TUNEL) in DKO embryos, indicating widespread apoptosis (Figure 2b). In summary, global deletion of *E2f7* and *E2f8* resulted in embryonic lethality by E11.5 and a spectrum of embryonic defects impacting the vasculature and cell survival.

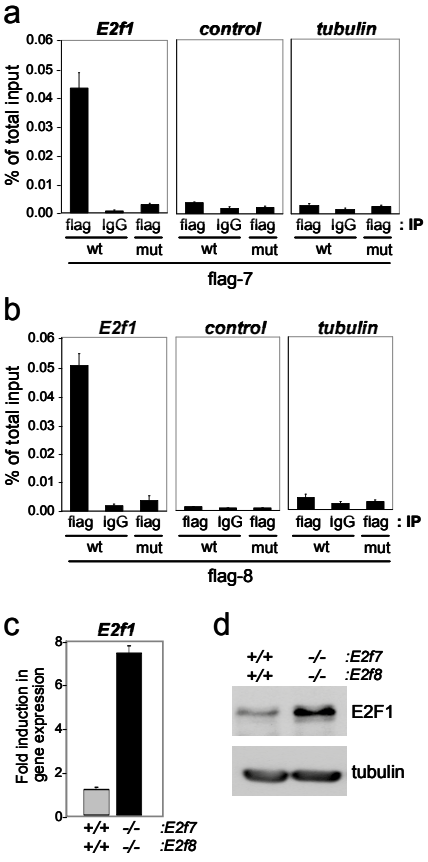


Figure 3. ***E2f1* is a direct target of *E2F7* and *E2F8*.** (a) Chromatin from cells overexpressing wild type flag-E2F7 (wt) or flag-E2F7DBD mutant (mut) was immunoprecipitated (IP) with anti-flag or IgG control antibodies. Immunoprecipitated DNA was amplified using primers specific for the *E2f1* promoter (*E2f1*), irrelevant sequences in the exon 1 of *E2f1* and in the *tubulin* promoter (*control* and *tubulin*, respectively). Quantitive PCR was performed in triplicate and cycle numbers were normalized to 1% of the input DNA. (b) Similar ChIP experiments were performed for E2F8. (c) Expression of *E2f1* in MEFs was measured by quantitive RT-PCR. (d) Western blot analysis of MEFs using antibodies specific for E2F1. Tubulin specific antibodies were used as an internal loading control.

E2f1 is a Direct Target of E2F7 and E2F8

E2F7 and E2F8 are believed to act as transcriptional repressors (Lammens et al., 2009). As a first step towards understanding the mechanism of how E2F7 and E2F8 function, we utilized chromatin immunoprecipitation (ChIP) assays to assess the ability of E2F7 and E2F8 to bind known E2F target promoters, such as *E2f1*. To this end, chromatin from cells overexpressing flag-E2F7 or flag-E2F8 was immunoprecipitated with antibodies specific for the flag epitope. Quantitive PCR assays showed that E2F7 and E2F8 were specifically recruited to the E2F binding sites on the *E2f1* promoter but not to the irrelevant sequences of *E2f1* (control) or to the *tubulin* promoter (Figure 3a, 3b). This recruitment was specific, since IgG antibodies

failed to immunoprecipitate *E2f1* promoter sequences. Moreover, anti-flag antibodies failed to immunoprecipitate the *E2f1* promoter from cell lysates expressing mutant forms of E2F7 or E2F8 proteins that are incapable of binding DNA.

To determine whether the recruitment of E2F7 and E2F8 to the *E2f1* promoter had any functional consequence on its expression, we examined E2F1 mRNA and protein levels in mouse embryo fibroblast (MEF) cells deficient for both *E2f7* and *E2f8*. Analysis of E2F1 expression in DKO cells showed a significant increase in its mRNA and protein levels relative to control MEFs (Figure 3c, 3d). Together, these results suggest that E2F7 and E2F8 directly occupy the *E2f1* promoter and repress its expression.

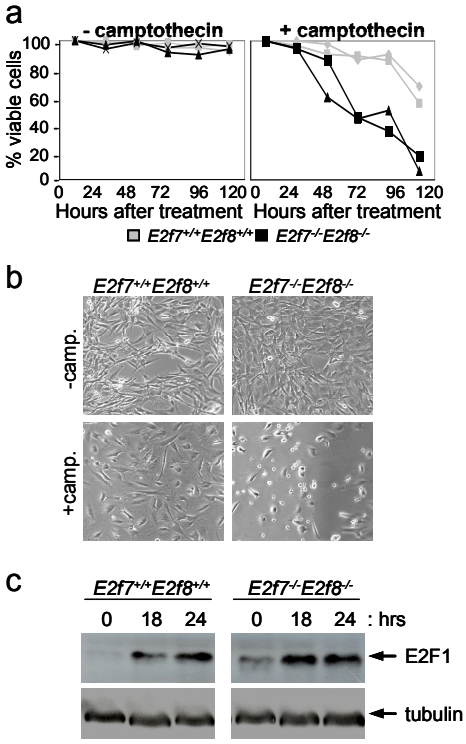


Figure 4. MEFs deficient in *E2f7* and *E2f8* are hypersensitive to DNA-damage induced apoptosis. (a) MEFs with the indicated genotypes were mock treated with DMSO (- camptothecin) or with camptothecin (+ camptothecin). The percentage of viable cells at the indicated time points are presented. (b) Light microscopy images of MEFs treated as in (a). (c) Lysates derived from MEFs treated as in (a) were analyzed by Western blotting using E2F1 specific antibodies. Tubulin-specific antibodies were used as an internal loading control.

DKO Cells are Hypersensitive to DNA-damage Induced Cell Death

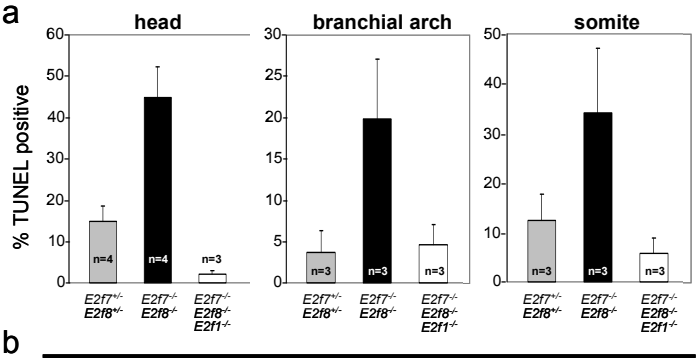
Given the observation that DKO embryos exhibited massive apoptosis and the fact that overexpression of E2F1 elicits apoptosis, especially under DNA-damage conditions (Stevens and La Thangue, 2004), we tested the sensitivity of DKO cells to camptothecin, a DNA-damage inducing drug. To this end, wild type and DKO MEFs were treated with camptothecin and cell viability was determined by trypan blue exclusion. Camptothecin induced a significant acceleration of cell death in DKO MEFs when compared to wild type MEFs (Figure 4a). Drug-treated DKO cells detached from tissue culture plates and

had the characteristic blebbing morphology of apoptotic cells (Figure 4b). We also evaluated the levels of E2F1 in drug-treated DKO MEFs by Western blotting. As expected, E2F1 protein accumulated to higher levels in drug-treated DKO cells than in similarly treated wild type cells (Figure 4c). These results indicate that E2F7 and E2F8 may attenuate DNA-damage induced apoptosis by preventing the aberrant accumulation of E2F1.

Induction of Apoptosis in DKO Embryos is Dependent on E2F1

The above findings prompted us to hypothesize that the apoptosis in DKO embryos may be mediated through induction of E2F1 *in vivo*. To test this possibility, *E2f7*$^{+/-}$*E2f8*$^{-/-}$ animals were bred with *E2f1*$^{-/-}$animals in order to generate *E2f7*$^{+/-}$*E2f8*$^{+/-}$*E2f1*$^{-/-}$mice. Timed pregnancies using these latter groups of mice were harvested at E9.5 and TUNEL assays were performed on triple knockout embryos (TKO, *E2f7*$^{-/-}$*E2f8*$^{-/-}$ *E2f1*$^{-/-}$). Indeed, TUNEL analysis revealed that *E2f1* was a physiologically relevant target, since its loss suppressed the massive apoptosis observed in DKO fetuses (Figure 5a). Together, these results identify E2F7 and E2F8 as a unique repressive arm of the E2F network that is critical for cell survival by controlling the E2F1 apoptotic pathway. Interestingly, however, inactivation of *E2f1* failed to prevent the death of DKO fetuses (Figure 5b), suggesting that apoptosis is not the leading cause of the observed

a



b

Genotypic analysis of *E2f1;E2f7;E2f8* embryos during development

	E2f1$^{+/+}$ *E2f7*$^{-/-}$*E2f8*$^{-/-}$	*E2f1*$^{+/-}$ *E2f7*$^{-/-}$*E2f8*$^{-/-}$	*E2f1*$^{-/-}$ *E2f7*$^{-/-}$*E2f8*$^{-/-}$	total
E9.5	-	2	5	46
expected	-	*5*	*6*	
E12.5	-	-	0(4)[a]	10
expected	-	-	*3*	

() number of dead embryos; Exact binomial test: [a] highly significant ($p<0.0007$)

Figure 5. Loss of *E2f1* suppresses apoptosis in *E2f7*$^{-/-}$*E2f8*$^{-/-}$ embryos. (a) Quantification of TUNEL-positive cells in the indicated tissue areas of *E2f7*$^{+/-}$*E2f8*$^{+/-}$, *E2f7*$^{-/-}$ *E2f8*$^{-/-}$ and *E2f1*$^{-/-}$ *E2f7*$^{-/-}$*E2f8*$^{-/-}$ fetuses are presented as the average ± SD percentage. (b) Genotypic analysis of embryos derived from *E2f1*$^{+/-}$*E2f7*$^{+/-}$ *E2f8*$^{-/-}$ intercrosses at the indicated stages of development.

lethality. In fact, TKO embryos harvested at E10.5 also exhibited dilated vessels and extensive hemorrhaging as seen in DKO embryos (data not shown).

Loss of E2f7 and E2f8 Leads to Profound Placental Defects

We then sought to determine the primary cause underlying the E11.5 lethality of DKO embryos and reasoned that identification of the tissues and cells in which E2F7 and E2F8 are most critical for embryo development/viability may provide more valuable insight into their physiological functions. To this end, we investigated a possible function of E2F7 and E2F8 in the placenta, since defective placentation often results in fetal growth retardation, and if sufficiently severe, may lead to mid-gestation lethality (Watson and Cross, 2005). Histological examination of hematoxylin and eosin (H&E)-stained $E2f7^{+/+}E2f8^{+/+}$ placenta sections showed that the placental architecture was sponge-looking and well organized by E10.5 (Figure 6a, left panels). In contrast, the architecture in DKO placentas was severely compromised (Figure 6a, right panels). *In situ* hybridization and immunofluorescence (IF) staining revealed that the double mutant placentas had lower expression levels of spongiotrophoblast- and giant cell-specific markers (Figure 6b and data not shown). Therefore, in addition to the defects previously characterized in DKO

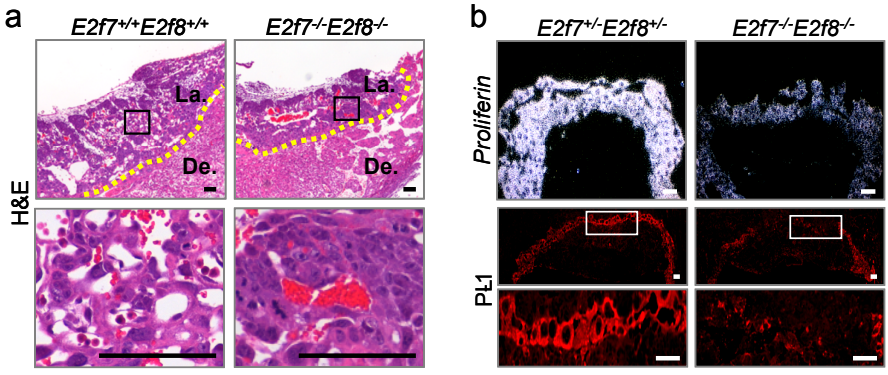


Figure 6. Profound placental defects in *E2f7*$^{-/-}$*E2f8*$^{-/-}$ embryos. (a) E10.5 placenta sections with the indicated genotypes were stained with H&E. The bottom panels represent higher magnification images of boxed areas demarcated in the top panels. De., Decidua; La., Labyrinth. (b) Top panels: RNA *in situ* hybridization analysis of *Proliferin*, a giant cell-specific marker on E9.5 placenta sections having the indicated genotypes. Middle and bottom panels: IF staining of E10.5 placenta sections with the indicated genotypes using antibodies specific for Placental Lactogen 1 (PL-1), a giant cell-specific marker. The boxed areas in middle panels are shown at higher magnification in the bottom panels. Scale bars, 100μm.

fetuses, these analyses show that double mutant placentas had extensive architectural abnormalities and severe differentiation defects.

Extra-embryonic Functions of E2F7 and E2F8 are Necessary and Sufficient for Embryo Survival.

The profound placental defects in DKO embryos encouraged us to rigorously evaluate the role of *E2f7* and *E2f8* in the placenta (extra-embryonic compartment). To this end, we took advantage of the conditional alleles of *E2f7* and *E2f8* (*E2f7*^{loxp} and *E2f8*^{loxp}) and different *cre* recombinases to specifically disrupt *E2f7* and *E2f8* function either in placental or fetal compartment (Figure 7a). *Cyp19-cre* transgenic mice express *cre* in all cell lineages of the mouse placenta, but not in the embryo proper (Wenzel and Leone, 2007). They were used to examine the consequence of inactivating *E2f7* and *E2f8* in the placenta. Genotypic analysis revealed that 8 out of 22 embryos with placenta-specific deletion (*Cyp19-cre; E2f7*$^{loxp/loxp}$*E2f8*$^{loxp/loxp}$) died at E10.5 and all were dead by E11.5 (Figure 7a, placental knockout, and data not shown). Importantly, these E10.5 live embryos showed a severe disruption of placental architecture that was associated with fetal

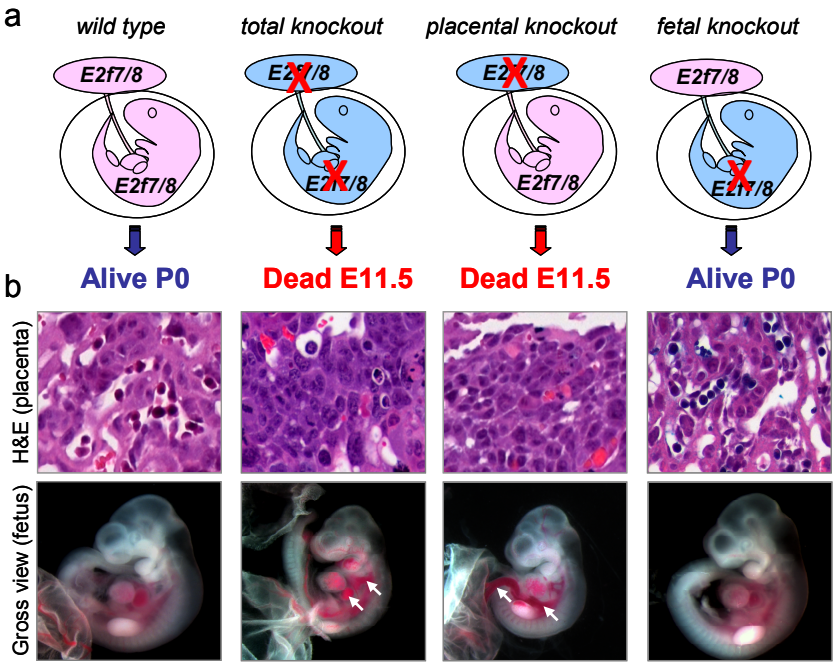


Figure 7. Extra-embryonic functions of *E2F7* and *E2F8* are both necessary and sufficient for embryo survival. (a) Embryo cartoon pictures of conventional and conditional knockout strategies. Total knockout (DKO), placental knockout (*Cyp19-cre*), and fetal knockout (*Sox2-cre*). The fetal viability was summarized as below. (b) Phenotypes of embryos with the indicated genotypes. Top panels: H&E staining of E10.5 placentas. Bottom panels: Gross appearance of E10.5 fetuses. Dilated blood vessels and hemorrhages are indicated by arrows. Note that the total knockout and placental knockout fetuses share similar phenotypes.

growth retardation, blood vessel dilation and hemorrhaging (Figure 7b, placental knockout). Thus, we conclude that extra -embryonic functions of E2F7 and E2F8 are critical for embryonic development and that their disruption causes the gross abnormalities and E11.5 lethality observed in DKO (total knockout) embryos.

We also utilized *Sox2-cre* transgenic mice to examine the consequence of inactivating *E2f7* and *E2f8* solely in fetal lineages. *Sox2-cre* mice express *cre* in the embryo proper, but not in extra-embryonic lineages (Hayashi et al., 2002). Strikingly, double mutant fetuses supplied with a wild type placenta (*Sox2-cre;E2f7*$^{loxp/-}$*E2f8*$^{loxp/-}$) were carried to term (Figure 7a, fetal knockout, and data not shown). These fetuses appeared normal at E10.5 and lacked any of the developmental phenotypes characteristic of similarly staged total knockout embryos (Figure 7b, fetal knockout, bottom panel). As expected, E10.5 placentas associated with these fetuses were morphologically normal (Figure 7b, fetal knockout, top panel). Together, these genetic analyses demonstrate that extra-embryonic functions of E2F7 and E2F8 are both necessary and sufficient for embryo survival.

E2F7 and E2F8 Control CellC ycle Progression in the Placenta.

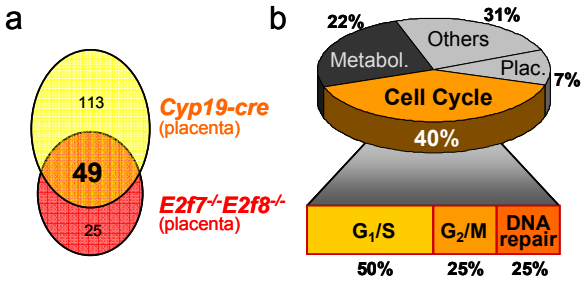


Figure 8. Genome-wide gene expression analysis of E10.5 placenta tissues. (a) Potential targets of E2F7 and E2F8 are presented by a two-set Venn diagram. Genes differentially upregulated (>2-fold, p<0.05) in placenta tissues from total knockout and placental knockout embryos are shown as colored circles. (b) Pie chart describing major gene function categories of 49 differentially expressed genes.

Knowing that the placenta is the critical tissue for E2F7 and E2F8 function allows us to further dissect cellular processes and molecular pathways regulated by these two novel E2Fs. To this end, we performed genome-wide gene expression analysis on E10.5 placentas from wild type, total knockout and placental knockout embryos. Venn diagram illustrated 49 genes that were commonly upregulated in total knockout

and placental knockout when compared to wild type (Figure 8a). Quantitive RT-PCR assays confirmed these results in a subset of selected targets (data not shown). Interestingly, functional annotation of the 49 gene-set revealed a high percentage of genes involved in the control of G_1-S and G_2-M specific events during cell cycle (Figure 8b).

The above molecular profiles prompted us to examine DKO placentas more closely for defects in cell cycle regulation. We first measured BrdU incorporation in the three main extra-embryonic lineages: giant cells (TG), spongiotrophoblast (SP) and labyrinth trophoblast (LT). This analysis revealed ectopic S phase entry in DKO TG and SP (Figure 9a, 9b). Surprisingly, we also detected many double mutant giant cells in mitosis, as measured by IHC with the mitotic marker P-H3 antibodies (Figure 9c, 9d). This was striking because giant cells normally reduplicate their genome but never undergo mitosis (Varmuza et al., 1988).

Together, systematic analyses of global gene expression profiles in placental tissues identified a transcriptional network repressed by E2F7 and E2F8 that control and coordinate G_1-S and G_2-M transitions. Disruption of this network results in ectopic DNA replication, aberrant mitosis, and led to profound placental abnormalities.

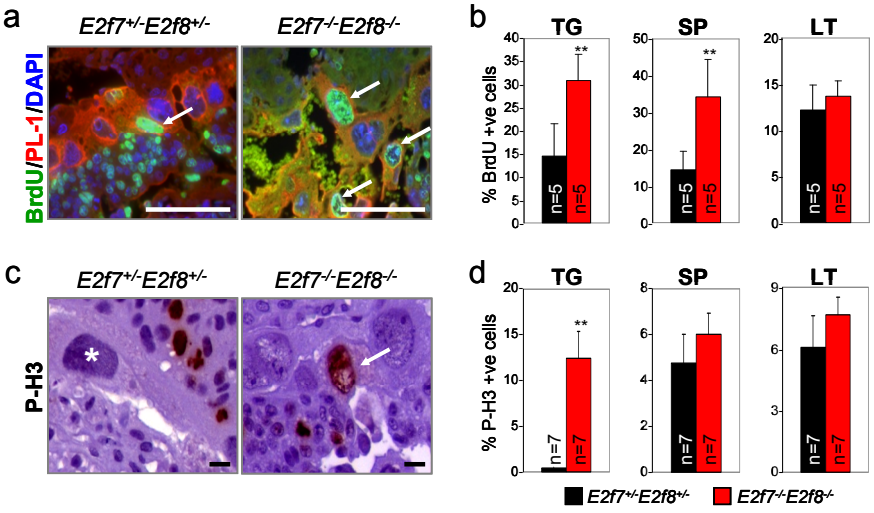


Figure 9. E2F7 and E2F8 regulate cell cycle progression in the placenta. (a) E10.5 placentas with the indicated genotypes were analyzed by BrdU/PL-1 double immunofluorescence staining with BrdU in green, PL-1 in red and DAPI in blue. Arrows indicate the BrdU/PL-1 double positive giant cells. (b) Percentages of BrdU positive cells were quantified for the indicated extra-embryonic lineages. (c) P-H3 immuno-biochemistry staining. Arrow points to an aberrant P-H3 positive giant cells. Star marks a P-H3 negative giant cell. (d) Percentages of P-H3 positive cells were quantified for the indicated extra-embryonic lineages. All data are presented as the average ± SD percentage of positive cells. n, number of placenta samples analyzed for each genetic group. Pairwise comparisons were evaluated by two-tailed Student's *t*-tests (** p<0.006). Scale bars, 100μm.

Discussion

Because of the intense interest in E2Fs as major regulators of the cell cycle and apoptosis, individual E2F family members, including *E2f1* through *E2f6*, have been extensively studied *in vivo* by knockout approaches in mice. Surprisingly, defects in embryos deficient for each of the known E2Fs are rather subtle (Attwooll et al., 2004). The virtual absence of cell proliferation and apoptotic defects in these embryos raised questions about the physiological importance of E2Fs, leaving the impression that these factors must either not be critical for the control of these processes *in vivo* or that there is sufficient functional redundancy among family members to accommodate for a deficiency in any single E2F. Here, we show that the disruption of *E2f7* or *E2f8* also had little consequence on mouse development. Their combined ablation, however, resulted in widespread apoptosis, profound placental and vascular defects, leading to embryonic death by E11.5.

In vitro and *in vivo* experiments described here provide clear-cut evidence in support of a role for E2F7 and E2F8 in the control of apoptosis. The mechanism of their action involves, at least in part, the regulation of *E2f1* expression. ChIP experiments showed that E2F7 and E2F8 are recruited to the *E2f1* promoter. Consistent with a role in repression of *E2f1*, genetic inactivation of *E2f7* and *E2f8* resulted in the accumulation of *E2f1* mRNA and a corresponding increase in its protein product. More importantly, the accumulation of E2F1 in DKO cells is of physiological significance since ablation of *E2f1* suppressed the widespread apoptosis observed in DKO embryos. Together, these results demonstrate a direct role for E2F7 and E2F8 in the control of fetal cell death and highlight the significance of the crosstalk between the E2F family members: E2F1, E2F7 and E2F8 (Figure 10).

In addition to their function in governing E2F1-dependnet apoptosis in the fetus, E2F7 and E2F8 also play an essential role in extra-embryonic lineages of the placenta. Their importance in these lineages was highlighted by conditional knockout experiments showing that their function in the placenta was both necessary and sufficient for embryo viability and development. Loss of *E2f7* and *E2f8* adversely affects cell cycle progression and impairs important cell lineage differentiation in the placenta (Figure 10). Thus, E11.5 lethality of DKO embryos could reflect the high sensitivity of placenta to subtle alterations in cell cycle control and restrictive requirement of fully developed placenta to maintain a successful pregnancy. Consistent with is idea, a recent survey of knockout mice with placental phenotypes revealed a prominent association of genes with functions in cell cycle regulation (Rossant and Cross, 2001; Watson and Cross, 2005). While the leading reasons for embryonic lethality in these cases are not well-defined, our data suggest that these embryos very likely die from placental dysfunction.

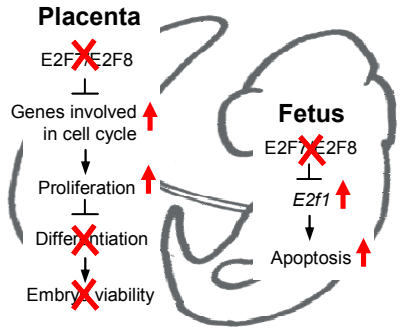


Figure 10. Current model of E2F7 and E2F8 function during embryo development.

The underlying mechanism of E2F7 and E2F8 action in controlling the timely cell cycle progression in the placenta involves the repression of a transcriptional network necessary for coordinating G_1-S and G_2-M transitions. Derepression of G_1-S related genes may be viewed to drive the ectopic DNA replication observed in DKO SP and TG cells, and derepression of G_2-M specific genes to contribute to the inappropriate nuclear division observed in double mutant TG cells. Importantly, a significant induction of proliferation was also observed in adult tissues lacking of *E2f7* and *E2f8* (data not shown). Therefore, E2F7 and E2F8 appear to widely function

as important repressors to suppress cellular proliferation. In this regard, it would be of great interest in the future to evaluatetheir potentialsuppress or function in cancer study.

In summary, using a combination of mouse genetics, bioinformatics, molecular biology and cell biology, this study clearly provides the first *in vivo* evidence for the physiological functions of E2F7 and E2F8, and successfully defined the critical cell lineages, cellular processes, molecular pathways affected by *E2f7* and *E2f8* loss. We conclude that E2F7 and E2F8-mediated repression controls apoptosis in the fetus and regulates cell cycle progression in the placenta (Figure 10). These findings not only add significant insights into how the 'total E2F' activity regulates cellular proliferation, apoptosis and differentiation in normal cells during development, not also strongly suggest a potential function of E2F7 and E2F8 as tumorsuppressors in cancers.

Methods and Materials

Mouse strains and genotyping -- The conventional and conditional *E2f7* and *E2f8* knockout mice, *Sox2-cre* and *Cyp19-cre* transgenic mice were maintained on a mixed background. Allele-specific primers used for *E2f7* and *E2f8* PCR genotyping were described previously (Li et al., 2008).

BrdU and TUNEL assays -- Pregnant females were injected with BrdU 30 min prior to harvesting. Embryos were fixed in formalin and paraffin embedded-sections were used for immunohistochemistry. Anti-BrdU antibody (MO-0744, DAKO) and Vectastain Elite ABC reagent (Vector labs) were used to detect BrdU incorporation according to the manufacturer's instructions. Apoptotic cells were detected using TUNEL (S7101, Chemican) assays, performed according to the manufacturer's protocol. All slides were counterstained with hematoxylin.

Chromatin immunoprecipitation (ChIP) assay -- The EZ CHIP^{TM} assay kit (Upstate Biotech) was used as described by the manufacturer. Briefly, human embryonic kidney 293 cells

overexpressing flag-E2F7 or flag-E2F8 were crosslinked and chromatin was sonicated. After pre-clearing, antibodies specific to flag or normal IgG (Oncogene) were added to each sample and incubated overnight. Antibody-protein-DNA complexes were recovered by addition of Salmon Sperm DNA/Protein G agarose slurry. Following extensive washing, the complexes were eluted and crosslinks were reversed. Finally, samples were treated with Proteinase K (Roche) and Rnase A (Roche) and purified through Qiaquick columns (Qiagen). Quantitive PCR of immunoprecipitated DNA was performed in triplicate and normalized using the threshold cycle number for the total input sample.

Quantitative RT-PCR -- Total RNA was isolated using Qiagen RNA mini-prep columns as described by the manufacturer, which included the optional DNase treatment before elution from the column. Reverse transcription (RT) of total RNA was performed using Superscript III reverse transcriptase (Invitrogen) and RNAse Inhibitor (Roche). Quantitative PCR was performed using a BioRad iCycler and reactions were performed in triplicate and relative amounts of cDNA were normalized to GAPDH.

Western blotting and antibodies -- Western blotting analyses were performed by standard procedures. Primary antibody was detected using horseradish-peroxidase-conjugated secondary antibodies and ECL reagents as described by the manufacturer (Amersham Biosciences). The following commercial antibodies were used: E2F1 (C-20, Santa Cruz), tubulin (T6199, Sigma).

Cell culture, proliferation and viability assay -- Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos. Immortalized cell lines ($E2f7^{loxp/loxp}E2f8^{loxp/loxp}$) were generated from primary MEFs using the standard 3T9 protocol and treated with retrovirus expressing *cre* recombinase using standard methods. To measure the sensitivity to apoptosis *in vitro*, MEFs were plated at approximate 30% confluency and treated with 20μm camptothecin

(Sigma) 24h after plating. Media was changed after 18h treatment, and samples were harvested at specific intervals as indicated. Cell viability was determined by trypan blue exclusion.

RNA in situ hybridization -- *In situ* hybridization was performed on E9.5 placenta sections using the standard protocol that was modified for paraffin-embedded sections. These modifications were deparaffinization in xylene and Proteinase K digestion. Radio-labelled probes were generated by *in vitro* transcription of *Proliferin* plasmid with either T7 RNA polymerase (Roche) using both ^{35}S-CTP and ^{35}S-UTP.

Genome-wide gene expression analysis --Total RNA was isolated using Qiagen RNA mini-prep columns according to the manufacturer's protocol. Global gene expression analysis was performed on Affymetrix Mouse Genome 430 2.0 arrays. Expression values were adjusted by quantile normalization and log2 transformation with RMAExpress, and data were analyzed with BRB-ArrayTools 3.7.0.

Histological analysis and immunostaining -- Placenta samples were fixed in formalin and embedded in paraffin. Standard hematoxylin and eosin (H&E) staining was used for general histopathological analysis. For Immunohistochemistry, slides were probed with primary antibodies specifically for Phospho-Histone 3 (P-H3, Ser10) (06-570, Millipore). Vectastain Elite ABC reagent (Vector labs) and DAB peroxidase substrate kit (Vector labs) were used in combination to detect P-H3 signals by following the manufacturer's instructions. Samples were counterstained with hematoxylin. Giant cells during S-phase transition were detected by double immunofluorescence staining using BrdU and PL-1 antibodies. Nuclear DNA was counterstained with DAPI.

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